Arch Virol (1990) 110: 195–212

Archives of Virology

Pathogenesis of Rift Valley fever in rhesus monkeys: role of interferon response

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Accepted October 24, 1989

Summary. Rhesus monkeys inoculated intravenously with Rift Valley fever (RVF) virus presented clinical disease syndromes similar to human cases of RVF. All 17 infected monkeys had high-titered viremias but disease ranged from clinically inapparent to death. Three (18%) RVF virus-infected monkeys developed signs of hemorrhagic fever characterized by epistaxis, petechial to purpuric cutaneous lesions, anorexia, and vomiting prior to death. The 14 remaining monkeys survived RVF viral infection but, 7 showed clinical signs of illness characterized by diminished food intake, cutaneous petechiae, and occasional vomiting. The other 7 monkeys showed no evidence of clinical disease. All monkeys had detectable serum interferon 24–30 h after infection, but 4 of 7 monkeys that did not develop clinical illness had serum interferon titers within 12 h after infection. In lethally infected macaques, indices of hepatic function and blood coagulation were abnormal within 2 days, implicating early pathogenetic events as critical determinants of survival. Serum transferase values were elevated in proportion to severity of clinical disease and outcome of infection. Both myocardial damage and laboratory evidence consistent with disseminated intravascular coagulation were present in fatal infections. All surviving monkeys developed neutralizing antibodies to RVF virus 4–7 days after infection, and this coincided with termination of viremia. Two fatally infected monkeys were viremic until death on days 6 and 8, and the third cleared viremia on day 5 and developed antibody on day 6 but died on day 15. There was a significant correlation between a delayed interferon response and mortality, suggesting that the early appearance of interferon was influential in limiting the severity of disease.

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Introduction

Human infection with Rift Valley fever (RVF) virus was recognized in 1931 and characterized as a transient undifferentiated febrile illness [1]. Subsequently, ocular disease, hemorrhagic fever, and encephalitis were found to occur in a small proportion of patients [2–6]. Patients with the viral hemorrhagic fever (VHF) syndrome develop hemorrhagic manifestations, icterus, and shock, with death frequently occurring within 5–10 days of disease onset. Laboratory data are limited but have shown elevated serum transaminases, hyperbilirubinemia, thrombocytopenia, and prolonged clotting parameters in affected patients [4, 6]. The pathogenesis of this syndrome is unknown, as is the reason for the occurrence of VHF in some infections but not in the majority. Certain strains of inbred rats provide a model for some of the diverse human responses to RVF virus infection, but the VHF syndrome has not been reported in RVF-infected rats. We have seen, as others have reported, that rhesus macaques inoculated with RVF virus also mimic human disease, usually presenting a nonfatal, febrile illness with viremia and focal hepatic necrosis [7–9]. However, during our studies with RVF-infected monkeys while investigating a non-human primate model for human RVF we have observed that RVF virus can induce VHF in approximately 20% of experimentally infected rhesus monkeys [10]. We therefore believe the rhesus monkey provides a realistic model with which to study human RVF and VHF, allowing serial observation of many parameters which are not practical or possible in the rat model. Understanding the mechanisms of disease is of intrinsic interest and is made more pressing by the recognized capacity of the virus to cause extensive epidemic disease and by the lack of practical prophylactic and therapeutic measures to combat viral infection.

Rift Valley Fever virus, a member of the genus *Phlebovirus* of the family *Bunyaviridae*, has been shown to be sensitive to interferon (IFN) in vitro. Furthermore, there are indications that IFN may be important in determining the outcome of rodent infections with RVF virus in vivo [11–13]. We have also observed that a therapeutic regimen of recombinant or bacteria-derived alpha interferon initiated 24 h prior to RVF virus inoculation prevented or greatly diminished viremia and clinical disease in rhesus macaques [14]. Therefore, we have undertaken more detailed studies on infected macaques, concentrating on the role of host IFN and antibody responses in regulating the outcome of infection.

Materials and methods

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Virus

The Zagazig 501 (ZH 501) strain of RVF virus, provided by Dr. James Meegan, U.S. Naval Medical Research Unit No. 3, Cairo, Egypt, was originally isolated in Egypt from a fatal human case of hemorrhagic fever. The virus was reisolated from human serum in diploid fetal rhesus lung cells (DBS-FRhL-2) and passaged a second time in these cells before use. The virus stock containing 5×10^7 plaque-forming units (PFU)/ml was stored at $-70\,^{\circ}C$ in Eagle's minimal essential medium supplemented with 10% heat-inactivated (56 °C for 30 min) bovine fetal serum and antibiotics (200 U/ml penicillin and 50 μg/ml streptomycin). Dilutions were prepared in Hank's balanced salt solution (HBSS) buffered to pH 7.4 with HEPES and containing 0.1% bovine serum albumin.

Experimental design

Seventeen (14 males, 3 females) healthy, adult rhesus monkeys (*Macaca mulatta*), weighing 5–10 kg, and negative for neutralizing antibody to RVF virus, were individually caged in a P-3 biological containment laboratory. These monkeys were each inoculated intravenously with 1.0 ml containing 1×10^5 PFU of ZH-501 strain of RVF virus and served as infected control monkeys for a series of four experiments investigating the efficacy of recombinant and bacteria-derived interferons in the prophylaxis and therapy of RVF in the rhesus monkey model [14]. Data were tested for heterogeneity and, when no differences were found, the four experiments were pooled for further analysis.

During the studies, each of the monkeys received intramuscular injections of sterile diluent in lieu of the experimental drug being tested. Monkeys were sedated with Ketamine® (Parke, Davis and Co., Detroid, MI) and 7–10 ml of whole blood was obtained by femoral or saphenous venipuncture. Monkeys were observed daily and blood samples collected 24 h prior to virus inoculation, immediately prior to virus inoculation, daily through postinoculation day (PID) 10, and periodically thereafter. An additional blood sample was collected 6 h after each sterile diluent injection. A complete necropsy was done on monkeys that died, and tissues were collected for both virus assay, and in 10% neutral formalin for histopathologic examination.

Virus assay

Serum for virus assay was separated from peripheral blood by centrifugation at $1,500 \times g$ and stored at $-70\,^{\circ}C$ until assayed. Tissues from fatally infected monkeys were prepared as 10% (w/v) homogenates in HBSS. After clarification by centrifugation, the supernatants were harvested and stored at $-70\,^{\circ}C$. Serial 10-fold dilutions of each serum and supernatant were assayed for RVF virus by counting plaques under agarose in 16 mm diameter Vero cell monolayers as described by Anderson et al. [15].

Plaque-reduction neutralization test (PRNT)

Serum neutralizing antibody titers were determined according to the method previously described [15]. The highest dilution of serum reducing 80% of the input virus was used as the endpoint for virus-neutralization titers ($PRNT_{80}$).

Hematologic and serum enzyme analyses

Whole blood in EDTA and serum were collected for hematologic examination and serum enzyme determinations. Total and differential WBC, hemoglobin, hematocrit (HCT), and platelet counts were done with an ELT-8/ds (Ortho Diagnostics, Westwood, MA) laser-based hematologic analyzer. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatine kinase (CK), MB fraction of CK (CK-MB), γ-glutamyltransferase (GGT), α-hydrobutyrate dehydrogenase [α-HBDH], and

lactic dehydrogenase (LD) values were determined with a Multistat III Plus (Instrumentation Laboratories, Lexington, MA) centrifugal chemical analyzer in the absorbance mode.

Interferon assay

Serum IFN was quantitated on IFN-α sensitive Madin-Darby bovine kidney (MDBK) cells and IFN-α, -β, and -γ sensitive human lung cancer (A 549) cells in a microtiter, dye-uptake, cytopathic effect (CPE)-inhibition assay [16–21]. In brief, duplicate, serial 2-fold dilutions of each specimen were made in 96-well microtiter plates. Approximately 3×10^4 freshly trypsinized cells were inoculated into each well. The plates were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. The cells were then challenged with 3,000 PFU/well of either vesicular stomatitis virus (MDBK) or encephalomyocarditis virus (A 549 cells). The virus-infected cell monolayers were incubated at 37 °C for an additional 30–36 h, until there was 100% CPE in the untreated control cell monolayers. Neutral red dye, diluted in Earle's balanced salt solution to a concentration of 0.1 mg/ml, was added to each well; the plates were incubated for an additional 2 h, washed three times with phosphate-buffered saline, and the dye eluted by an acidified alcohol buffer (Sorenson's citrate I and ethanol 1 : 1 v/v, pH 4.2). The optical density of each well was determined with a microplate spectrophotometer (Dynatech Laboratories, Inc., Alexandria, VA) at a setting of 570 nm. Appropriate IFN reference and laboratory standards were incorporated into each assay.

Interferon titers were determined as the reciprocal of the highest dilution of sample to protect 50% of the cells from CPE. Fifty percent protection was determined to be that optical density value representing one-half (+2 SD) of the mean optical density of uninfected cell control wells. Interferon titers were expressed as units/ml (U/ml) of serum standardized against the National Institutes of Health (NIH) IFN standards (Catalog Nos. Ga 23-902-530 and Gg 23-901-530, Division of Research Resources, National Institutes of Health, Bethesda, MD).

Serum IFN-α was identified on the IFN-α-sensitive MDBK cells, MDBK cells are insensitive to human IFN-β and -γ and we have observed that non-human primate IFN-γ and NIH IFN-β and -γ reference standards do not inhibit the cytopathic effect of VSV in MDBK cells [20]. In the present study, samples which were positive for interferon, were positive by both assay systems though titers were routinely higher in the MDBK cells than in the A 549 cells. In no case was IFN detected in A 549 cells and not MDBK cells. The IFN titers presented are those obtained from MDBK cells.

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey multiple comparison tests were used for analysis at the 0.05 level. The relationship of temporal appearance of IFN to morbidity and mortality in RVF virus-infected monkeys was analyzed by the Fisher exact test at the 0.05 level.

Results

Clinical responses

Monkeys could be divided into three groups on the basis of course of disease after intravenous inoculation: fatally infected monkeys that developed severe clinical disease and died, clinically ill monkeys that survived, and monkeys that developed very mild or no apparent clinical illness and survived. Three male monkeys (3/17, 18%) developed severe clinical disease. Signs were first apparent

2–4 days after virus inoculation, and were characterized by anorexia, depression, vomiting, and weakness. Bleeding was manifested by petechial and purpuric skin lesions most noticeable on the face, ears, abdomen, and medial thigh. Monkey 5606 developed epistaxis on PID 8 which continued intermittently until the monkey was euthanatized on PID 15 due to anorexia, depression, and debilitation. Monkey 4880 died on PID 8, and monkey 9 C 87 developed epistaxis on PID 5 which continued through PID 6 when the animal was euthanatized.

Seven monkeys (5 males, 2 females, 41%) survived experimental RVF virus infection, but developed clinical signs characterized by diminished appetite, cutaneous petechial hemorrhages on the abdomen and medial aspect of the thigh, and occasional vomiting.

The remaining seven monkeys (6 males, 1 female) developed no apparent clinical illness other than a brief pyrexia coincident with peak viremia.

Body temperatures were monitored in only 11 (1 fatally infected, 5 clinically ill, and 5 non-ill) monkeys and all experienced a febrile response that paralleled the onset and disappearance of viremia. The highest rectal temperature recorded was 40 °C from fatally infected monkey 9 C 87 48 h after RVF virus inoculation. The surviving monkeys were systematically examined (including fundoscopy under anesthesia) for 60 days after RVF virus inoculation, and some were observed for up to 2 years, with no clinical evidence of postinfection encephalitis or retinal complications.

Viremia

All 17 monkeys were viremic 24 h after virus inoculation, with maximum viral titers detected between 30 and 48 h postinfection. Fatally infected and clinically ill monkeys exhibited significantly higher viremia titers of longer duration than the survivors ($P < 0.05$). The two fatally infected monkeys that died on PID 6 and 8 were viremic at the time of death. The third fatally infected monkey (5605) was aviremic after PID 5, yet became moribund and was euthanatized on PID 15.

All seven clinically ill monkeys that survived were viremic through PID 4. Four of these monkeys were viremic on PID 5, one was viremic on PID 6, and no viremia was detected after PID 6 (Table 2).

The seven surviving monkeys that showed no signs of clinical illness all were viremic through PID 3. Viremias of this group became undetectable earlier than those of the more seriously affected monkeys, with only two animals having detectable viremia on PID 5 (Table 3).

Neutralizing antibody

Among the fatally infected macaques, neutralizing antibody was detected only in the monkey euthanatized on PID 15 (Table 1). Antibody in that monkey was first detected on PID 8 (1:320), and by PID 15 had risen to 1:5,120. Neutralizing antibody responses of the other two groups were similar to each other in that disappearance of viremia coincided with the appearance of neu-

Table 1. Viremia (*VT*), neutralizing antibody (*PRNT*), and interferon (*IFN*) titers of lethally infected monkeys

	Monkey no.								
	4880			5606			9C87		
Day	VT[a]	PRNT[b]	IFN[c]	VT	PRNT	IFN	VT	PRNT	IFN
0	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10
0.25	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10
0.5	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10
1	5.8	<20	<10	5.8	<20	<10	7.4	<20	480
1.25	6.5	<20	160	6.8	<20	160	7.5	<20	480
2	6.7	<20	160	6.3	<20	160	7.0	<20	480
2.25	5.9	<20	40	5.5	<20	80	6.2	<20	480
3	5.7	<20	<10	5.2	<20	<10	5.7	<20	120
3.25	5.0	<20	<10	4.7	<20	<10	5.5	<20	60
4	5.0	<20	<10	4.4	<20	<10	5.1	<20	120
4.25	5.0	<20	<10	3.8	<20	<10	4.0	<20	120
5	4.3	<20	<10	3.3	<20	<10	3.9	<20	120
6	4.5	ND[d]	<10	<.7	ND	<10	4.2[e]	<20	120
7	4.7	ND	<10	<.7	ND	<10			
8	4.9[e]	<10	80	<.7	320	<10			
15				<.7[e]	5120	<10			

[a] Data expressed as $\log_{10}$ PFU/ml serum
[b] Data expressed as reciprocal of $PRNT_{80}$ antibody titer
[c] Data expressed as U/ml serum compared to NIH human reference standard Ga 23-902-530
[d] Not done
[e] Died or killed in a moribund condition

tralizing antibody. Occasionally, low-titered neutralizing antibody (1 : 20) was detectable in viremic sera 24 h before viremia became undetectable. Serum-neutralizing antibody was first detected as early as 78 h after virus inoculation in one of the monkeys that showed no signs of illness, and as late as PID 7 in one monkey (viremic on PID 6) from the clinically ill group (Tables 2 and 3).

Interferon

Among the lethally infected monkeys (Table 1), interferon was first detected at 24 h in 9C87 and persisted in high titer until death on day 6. In the other two non-survivors, interferon initially appeared at 30 h, but was undetectable by day 3 in spite of continuing viremia. One of these animals (4880) had a

Table 2. Viremia (*VT*), neutralizing antibody (*PRNT*), and interferon (*IFN*) titers of clinically ill surviving monkeys

	Monkey no.																				
	MO25			X662			490C			829			B7			T34			472B		
PID	VT[a]	PRNT[b]	IFN[c]	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN
0	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10
0.25	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10
0.5	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10
1	5.4	<20	10	5.2	<20	10	5.2	<20	60	5.6	<20	160	6.7	<20	120	6.7	<20	80	5.7	<20	60
1.25	6.0	<20	640	5.9	<20	320	6.7	<20	160	6.5	<20	240	6.7	<20	160	6.6	<20	60	6.1	<20	480
2	6.1	<20	320	6.4	<20	320	5.7	<20	120	5.3	<20	80	5.0	<20	40	6.7	<20	80	6.6	<20	480
2.25	5.7	<20	320	6.2	<20	80	6.2	<20	80	5.5	<20	60	5.6	<20	40	5.8	<20	40	5.8	<20	480
3	5.7	<20	160	6.0	<20	80	5.3	<20	40	5.4	<20	20	5.4	<20	10	5.2	<20	10	5.6	<20	240
3.25	5.2	<20	80	5.7	<20	40	5.0	<20	10	4.9	<20	10	5.4	<20	40	4.9	<20	10	5.5	<20	160
4	5.1	<20	10	5.6	<20	10	4.4	<20	20	4.4	20	<10	4.7	<20	20	3.8	<20	40	4.5	<20	120
4.25	<.7	<20	<10	<.7	<20	<10	4.4	<20	20	4.5	20	<10	4.6	<20	20	3.5	<20	40	3.5	<20	120
5	<.7	20	<10	<.7	20	<10	2.5	20	<10	2.7	20	<10	2.7	40	<10	2.8	<20	<10	2.4	<20	80
6	<.7	20	<10	<.7	20	<10	<.7	20	<10	<.7	20	<10	<.7	40	<10	2.1	20	<10	<.7	<20	40
7	<.7	80	<10	<.7	80	<10	<.7	320	<10	<.7	20	<10	<.7	320	<10	<.7	320	<10	<.7	20	40
10	<.7	320	<10	<.7	1280	10	<.7	1280	20	<.7	320	<10	<.7	1280	<10	<.7	320	<10	<.7	80	<10
14	<.7	320	<10	<.7	1280	<10	<.7	1280	<10	<.7	1280	<10	<.7	2560	20	<.7	2560	<10	<.7	320	<10
21	ND[d]	ND	ND	ND	ND	ND	<.7	1280	<10	<.7	2560	<10	<.7	2560	<10	<.7	2560	<10	<.7	1280	<10
28	ND	ND	ND	ND	ND	ND	<.7	1280	<10	<.7	5120	<20	<.7	1280	<10	<.7	10240	<10	<.7	10240	<10

[a] Data expressed as $\log_{10}$ PFU/ml serum
[b] Data expressed as reciprocal of $PRNT_{80}$ antibody titer
[c] Data expressed as U/ml serum compared to NIH human reference standard Ga 23-902-530
[d] Not done

Table 3. Viremia (*VT*), neutralizing antibody (*PRNT*), and interferon (*IFN*) titers of non-ill, surviving monkeys

	Monkey no.																				
	B3			P7046			B2			Y35			18434			532C			9C90		
PID	VT[a]	PRNT[b]	IFN[c]	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN	VT	PRNT	IFN
0	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10
0.25	<.7	<20	<10	<.7	<20	40	<.7	<20	<10	<.7	<20	<10	<.7	<20	<10	<.7	<20	80	<.7	<20	20
0.5	<.7	<20	<10	<.7	<20	80	<.7	<20	10	<.7	<20	<10	<.7	<20	<10	<.7	<20	120	<.7	<20	120
1	5.7	<20	10	5.5	<20	160	3.8	<20	80	4.3	<20	10	5.3	<20	80	7.1	<20	240	6.5	<20	120
1.25	6.3	<20	160	5.8	<20	320	4.5	<20	160	5.4	<20	40	5.6	<20	160	7.3	<20	240	6.4	<20	120
2	6.2	<20	160	6.0	<20	160	2.4	<20	20	4.5	<20	120	5.8	<20	120	6.6	<20	240	6.6	<20	240
2.25	5.3	<20	160	5.3	<20	160	2.7	<20	<10	3.5	<20	60	5.3	<20	60	6.3	<20	240	6.5	<20	240
3	3.9	<20	80	5.3	<20	80	1.6	<20	<10	<.7	<20	<10	3.9	<20	40	5.1	<20	160	6.0	<20	160
3.25	3.4	<20	10	4.6	<20	80	<.7	<20	<10	<.7	<20	<10	2.8	20	40	5.1	<20	160	5.4	<20	120
4	<.7	20	<10	<.7	20	10	<.7	20	<10	<.7	40	<10	<.7	80	<10	4.2	<20	80	3.7	<20	120
4.25	<.7	20	<10	<.7	20	<10	<.7	20	<10	<.7	40	<10	<.7	80	<10	3.4	<20	80	2.5	<20	120
5	<.7	20	<10	<.7	20	<10	<.7	20	<10	<.7	80	<10	<.7	80	<10	2.6	<20	120	1.8	<20	60
6	<.7	80	<10	<.7	80	<10	<.7	80	<10	<.7	160	<10	<.7	80	<10	<.7	20	80	<.7	20	60
7	<.7	320	<10	<.7	320	<10	<.7	320	<10	<.7	320	<10	<.7	320	<10	<.7	40	40	<.7	80	40
10	<.7	1280	<10	<.7	320	<10	<.7	320	<10	<.7	640	<10	<.7	320	<10	<.7	320	<10	<.7	320	<10
14	<.7	1280	<10	<.7	1280	<10	<.7	1280	120	<.7	2560	<10	<.7	1280	<10	<.7	320	<10	<.7	320	<10
21	ND[d]	ND	ND	ND	ND	ND	ND	1280	<10	<.7	5120	<10	<.7	5120	<10	<.7	1280	<10	<.7	2560	<10
28	ND	ND	ND	ND	ND	ND	ND	1280	<10	<.7	5120	<10	<.7	10240	<10	<.7	1280	<10	<,7	5120	<10

[a] Data expressed as $\log_{10}$ PFU/ml serum
[b] Data expressed as reciprocal of $PRNT_{80}$ antibody titer
[c] Data expressed as U/ml serum compared to NIH human reference standard Ga 23-902-530
[d] Not done

recurrence of interferonemia (80 U/ml) in a serum sample obtained on day 8, the day of death.

Serum IFN was first detected at 24 h after RVF virus infection in all seven surviving monkeys that showed clinical illness. Titers at this time ranged from 10–160 U/ml of serum. Peak of mean serum IFN titers was seen at 30 h post-infection and titers gradually declined to undetectable levels by PID 5, except for one monkey that maintained a serum interferonemia through PID 7 (Table 2).

Serum IFN was first detected 6 h after RVF virus infection in 3 of 7 monkeys that showed no signs of illness. At 12 h after infection, a fourth monkey had detectable serum IFN, and by 24 h after infection, all seven monkeys of this group had detectable serum IFN titers ranging from 10–240 U/ml of serum. At this time, the waning of serum IFN paralleled that of the survivors showing clinical signs. By PID 4, five monkeys had no detectable serum IFN, but the two other animals had serum IFN titers through PID 7 (Table 3).

Statistically there was no significant difference in the temporal appearance of IFN and clinical outcome between the non-ill and ill groups ($P = 0.100$). However, in spite of the small size of each group, there were significant differences in the initial appearance of serum IFN in the non-ill or ill groups compared to the fatally infected group ($P = 0.042$ and 0.049).

The disappearance of viremia and serum IFN coincided with the appearance of neutralizing antibody in all but the fatally infected monkeys.

Hematologic and serum enzyme analyses

The hematologic data are shown in Fig. 1. Lethally infected monkeys had a pronounced leukocytosis, which reached 2–3 times the mean baseline value (6.7×10^3 cells/dl), and which either remained elevated until death or returned to baseline values just prior to death. Surviving monkeys with clinical illness responded to RVF virus infection with a transient leukocytosis reaching as high as 16.9×10^3 cells/dl within 24–48 h after infection, followed by a brief leukopenia and a return to baseline values (7.4×10^3 cells/dl) 4–7 days after infection. Unfortunately, differential white cell counts were not done on all monkeys, however, those that were done reflected a pronounced lymphopenia (mean 2.2×10^3 cells/dl falling to 9×10^2 cells/dl) concurrent with neutrophilia (mean 4.5×10^3 cells/dl rising to 8.7×10^3 cells/dl). The RVF virus-infected monkeys that did not develop clinical illness had a mild, neutrophilic leukocytosis 24–48 h after infection, which returned to baseline values 3–4 days after infection.

Hematocrit values decreased in all three groups relative to the degree of clinical illness observed. The fatally infected monkeys' HCT values dropped from a mean of 43% to 19%, 22%, and 29% on their respective days of death. The nonfatally infected groups' mean HCT value dropped from 42% on PID 0 to 34% on PID 14, before gradually returning to within baseline limits. The

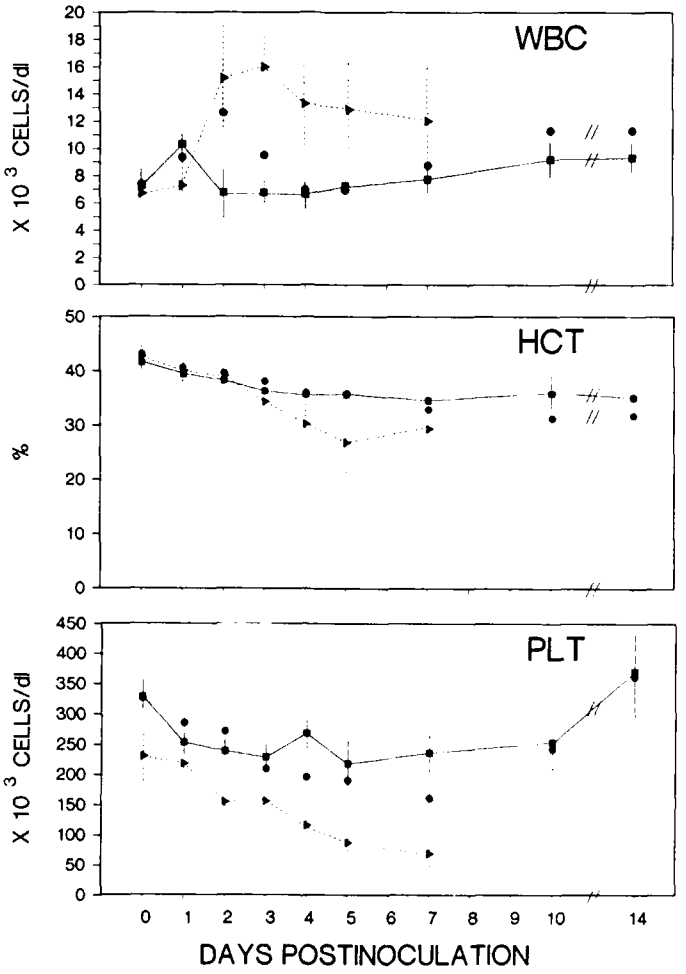

Fig. 1. Mean (± SEM) white blood cell (*WBC*), hematocrit (*HCT*), and platelet (*PLT*) counts in Rift Valley fever virus-infected rhesus monkeys. ▲ Fatally infected monkeys (deaths on days 6, 8, and 15); ● clinically ill surviving monkeys; ■ non-ill surviving monkeys

depressed HCT values may, in part, reflect blood collection for testing. During the first 5 days of the study, 7–10 ml of blood was collected from each monkey twice daily.

After RVF virus infection, platelet counts in all three groups decreased in proportion to severity of disease. Platelet counts in the lethally infected monkeys dropped from a mean of 230,000 cells/dl to a mean of 68,000 cells/dl prior to death. Platelet counts of the ill monkeys decreased from a baseline mean of 327,000 cells/dl to a mean of 163,000 cells/dl on PID 7 before returning to baseline values. Mean platelet counts of the survivors without clinical illness decreased from a mean of 326,000 cells/dl to 219,000 cells/dl on PID 5 before returning to baseline values.

Serum aminotransferase (AST, ALT, GGT) values were measured to assess liver damage. The fatally infected monkeys had peak mean increases in AST,

Table 4. Mean (± SEM) serum enzyme values in Rift Valley fever virus-infected rhesus monkeys

		Postinoculation day							
		0	1	2	3	5	7	10	14
Fatal	AST	36 ± 6	64 ± 8	1568 ± 32	1107 ± 232	4882 ± 2763	893 ± 31	NA	NA
	ALT	38 ± 4	55 ± 2	430 ± 44	460 ± 44	1490 ± 889	1004 ± 126	NA	NA
	LDH[a]	283	421	3669	4290	5841	NA[b]	NA	NA
	CK	89 ± 7	805 ± 102	4080 ± 464	5957 ± 913	7420 ± 2305	429 ± 337	NA	NA
	HBDH[a]	515	781	4932	5955	6969	NA	NA	NA
	(g)-GT	41 ± 3	45 ± 3	222 ± 26	220 ± 5	166 ± 7	142 ± 32	NA	NA
	CKMB[a]	20	43	95	105	310	NA	NA	NA
Ill	AST	33 ± 2	72 ± 13	525 ± 357	270 ± 62	155 ± 34	140 ± 47	40 ± 4	38 ± 5
	ALT	35 ± 7	48 ± 6	205 ± 43	181 ± 31	195 ± 54	126 ± 47	49 ± 8	43 ± 10
	LDH	288 ± 54	457 ± 90	3705 ± 485	4334 ± 690	3136 ± 1163	1616 ± 688	513 ± 78	293 ± 37
	CK	105 ± 17	1249 ± 209	5753 ± 1106	6730 ± 1597	4031 ± 1179	1062 ± 514	94 ± 16	70 ± 17
	HBDH	444 ± 76	651 ± 99	4425 ± 548	5311 ± 773	3956 ± 1165	2241 ± 742	906 ± 117	539 ± 75
	(g)-GT	45 ± 6	44 ± 6	111 ± 31	129 ± 36	104 ± 28	63 ± 14	57 ± 9	61 ± 7
	CKMB	26 ± 12	97 ± 48	91 ± 24	90 ± 23	142 ± 66	36 ± 20	14 ± 9	15 ± 9
Non-ill	AST	30 ± 2	54 ± 7	295 ± 64	152 ± 26	83 ± 25	47 ± 8	34 ± 6	27 ± 3
	ALT	33 ± 5	40 ± 4	199 ± 59	165 ± 51	93 ± 22	49 ± 11	38 ± 7	29 ± 6
	LDH	248 ± 29	284 ± 60	1297 ± 371	1139 ± 384	893 ± 339	522 ± 81	418 ± 46	295 ± 63
	CK	97 ± 21	1305 ± 283	3413 ± 827	2582 ± 607	931 ± 389	183 ± 54	102 ± 38	49 ± 9
	HBDH	467 ± 91	469 ± 51	1756 ± 710	1538 ± 748	1039 ± 444	850 ± 170	670 ± 135	495 ± 114
	(g)-GT	39 ± 3	37 ± 4	53 ± 9	58 ± 16	51 ± 12	41 ± 5	45 ± 5	46 ± 8
	CKMB	17 ± 1	85 ± 51	74 ± 20	47 ± 5	61 ± 33	25 ± 10	47 ± 20	24 ± 10

[a] Results of 1 monkey only
[b] Data not available

ALT, and GGT of approximately 100-fold, 25-fold, and 5-fold, respectively, over their PID 0 baseline values. Enzyme values in surviving monkeys were considerably lower and peaked sooner. Sick survivors had somewhat higher values than those without apparent illness (Table 4).

Serum CK values were elevated in all three groups of monkeys by 24 h postinoculation. The fatally infected and clinically ill monkeys showed similar increases in serum CK. Serum CK values of the clinically ill survivors returned to baseline by PID 10. The monkeys with no clinical signs had lesser increases in their serum CK and a return to near baseline values (97 IU/l) by PID 7.

Serum CK-MB was determined in one fatally infected monkey (9 C 87) on the day of death, PID 6. It was compared to that of three clinically ill and three non-ill survivors that had the highest serum CK values after RVF virus infection. Monkey 9 C 87 had a 20-fold increase in serum CK-MB, whereas the ill and non-ill survivors had 7- and 4-fold increases, respectively. By PID 7, serum CK-MB had returned to baseline values (21 IU/l) in the survivors.

Serum LDH and HBDH values showed similar patterns to those observed in CK assays. Sick monkeys had the greatest increases, and the least changes from baseline were seen in those with no clinical signs.

There was no significant difference in serum BUN values among the three groups of monkeys.

Postmortem examination of fatally-infected rhesus monkeys

On postmortem examination, each fatally infected monkey had multiple cutaneous petechial to purpuric hemorrhages; ecchymotic hemorrhage in the peritesticular connective tissue; and a swollen, congested liver. Monkey 9 C 87 had massive extravasation of blood into the subcutaneous tissues over the left arm and axillary region.

Microscopically, the salient pathologic changes were in the livers of all three monkeys. Extensive moderate to severe centrolobular and midzonal coagulative necrosis occurred in all lobules. The necrotic foci ranged in size from a few hepatocytes to areas exceeding 2 mm in diameter. A portal cellular infiltrate involving vascular walls, connective tissue, and adjacent hepatic plates was observed in several portal triads. Lymphoplasmacytes dominated the infiltration; however, macrophages and granulocytes were distributed among the lymphoid cells. Moderate, multifocal, epicardial, and interstitial hemorrhage in the heart, and multifocal necrosis and myocarditis were observed in monkeys 4880 and 5606. Mild to moderate perivascular cuffing of primarily lymphoplasmacytic cells was observed surrounding most vessels in the choroid plexus and epididymis of monkey 9 C 87 killed on PID 6. Monkey 4880, dead on PID 8, had a multifocal, acute, encephalitis in the cerebral cortex characterized by nodular aggregates of neutrophils in association with mild necrotic changes of neurons. Monkey 5606, killed on PID 15, had a mild, nonsuppurative, multifocal, perivascular encephalitis, primarily of lymphoplasmacytic cells.

Table 5. Viral titers[a] of organs from fatally infected monkeys

Organ	Monkey no.		
	9C87	4880	5606
Serum	4.2	4.9	< 0.7
Spleen	6.1	5.1	2.3
Liver	6.9	5.0	< 1.4
Adrenal	4.7	4.7	< 1.4
Kidney	4.3	4.4	< 1.4
Brain	1.3	3.8	< 1.4
Heart	< 1.4	3.6	< 1.4
Lung	2.7	3.4	< 1.4
Epididymis	2.9	2.6	< 1.4
Skin	2.3	< 1.4	< 1.4
Mesenteric L.N.	4.2	ND[b]	2.9
Bone marrow	5.2	ND	ND
Aorta	2.8	ND	ND

[a] Expressed as $\log_{10}$ PFU/g tissue
[b] Not done

A hematoma was located in a lobe of the thyroid gland of monkey 9C87. A fibrin thrombus occluded a small vessel in the perithyroid connective tissue of this lobe.

Tissue viral titers

The concentrations of infectious virus recovered from tissues of the three fatally infected monkeys were determined (Table 5). Most tissues examined from monkeys 4880 and 9C87 contained virus, with the highest concentrations found in the spleen and liver. Virus was recovered from only the spleen and mesenteric lymph node of monkey 5606.

Discussion

Most human infections with RVF virus result in a temporarily debilitating febrile illness of a few days duration, followed by convalescence and full recovery within 2–3 weeks after the febrile period. High-titered viremias are present during the febrile period, followed by the development of neutralizing antibodies 4–10 days after the onset of symptoms. A minority, probably about 1% or less of human cases, are complicated by encephalitis, retinal lesions, or VHF. The determinants of these different syndromes are unknown. In the case of VHF, underlying hepatic disease has been suggested as a factor, but is not a prerequisite. The genesis of the hemorrhagic manifestations in patients with VHF is not understood, although there are anecdotal reports of abnormal coagulation tests and thrombocytopenia [22, 23].

Since an intravenous inoculation with the ZH 501 strain of RVF virus had produced a VHF syndrome in 3 of 15 rhesus monkeys, we elected to explore the role of IFN in pathogenesis of RVF in macaques [10]. Evidence suggesting that IFN is important in the pathogenesis of rodent RVF virus infections, and our interest in the use of recombinant IFN in the prophylaxis of RVF, also led us to measure the dynamics of IFN production. In the studies reported here, infection of rhesus monkeys led to significant viremia and abnormal liver function tests in each of the 17 animals inoculated. Seven animals (41%) had no clinical manifestations; 7 were obviously ill, but survived; 3 (17%) had fatal VHF. None of the animals had significant neurological signs, although two of the dying rhesus monkeys had minor histological abnormalities of the central nervous system. Neither systematic ophthalmoscopic examination of survivors nor nistopathological studies of necropsy material indicated ocular involvement.

The severity of disease correlated with the intensity and duration of viremia. Clinically well animals were viremic for 3–5 days, and sick survivors for 4–6 days. One fatally infected animal terminated his viremia by day 5, but the other two had viremia in excess of $4.0 \log_{10}$PFU/ml at the time of death on days 6 and 8. Viremia terminated contemporaneously with the appearance of neutralizing antibodies. Indeed, low-level neutralizing antibodies (1 : 20–40) often could be measured in sera collected on PID 4 or 5 when viremia was waning. This pattern has been seen in rodent infections, and serum antibody has been implicated as the major virus-specific immunological mechanism in recovery [12, 15]. The close temporal correlation of neutralizing antibody appearance with clearance of viremia in this study, and the very effective prophylaxis of rhesus monkey RVF virus infection with passively transferred antibody [10] suggest that this may be the case in macaques as well.

Virus induction of interferon is a complex process dependent upon inducer and target cell type. Viral interferon induction is generally considered a "late" response, with serum interferon appearing several hours to days after virus inoculation [19, 24]. Studies have shown that in some diseases an early interferon response can be predictive of the outcome. A correlation between levels of vesicle interferon and the course of disease was shown in herpes zoster infection [25]. Patients could be separated based on the later rise of vesicle interferon in those with disseminated disease. The authors hypothesize that a prompt immunological response in conjunction with an early local interferon response will contain the infection. The duration of high serum interferon titers may also have prognostic value. In humans infected with Junin virus, high levels of serum interferon were found during the first week after onset of symptoms. In those patients which survived, serum IFN titers decayed during the late stages of the disease, but in patients with a fatal outcome, extremely high interferon titers persisted and may have enhanced the disease process [26]. Thus it seems that in some diseases interferon may, in fact, be detrimental. Jacobson et al. [27] observed that interferon inducers actually increased pathogenesis in lymphocytic choriomeningitis virus infected mice. Furthermore, Pfau et al. [28] showed that

the lethal effect of endogenous interferon in LCM virus-infected adult mice could be prevented with anti-IFN globulin.

Bioassays for IFN measure the bioactivity in a specimen based on the inhibition of some component of virus replication. A variety of assays are available and we have standardized a simple and convenient cpe-inhibition assay to facilitate testing of large numbers of human and non-human primate sera quickly and reproducibly. The IFN bioassay used in the present study monitors the appearance and subsequent disappearance, in the serum of RVF virus-infected monkeys, of a serum factor capable of inhibiting the cytopathic effect of VSV in MDBK cells. These cells are sensitive to human leukocyte interferon and insensitive to human beta and gamma interferons [20]. The simultaneous detection of the viral inhibitory factor in an assay system using A 549 cells, which are sensitive to all human interferons, at a lower titer than the MDBK cells is highly suggestive that the viral inhibition is due to IFN-α [21].

Interferon appears to play an important role in the outcome of RVF viral infection. Rift Valley fever virus is sensitive in vitro to IFN-α and the outcome of RVF virus infection in rodents and monkeys appears to be regulated by IFN and serum antibody [11–14, 29]. In this study there was evidence that the early appearance of serum IFN was a contributory factor in limiting viremia and preventing clinical disease in the non-ill gr . Early samples were taken at 6, 12, 24, and 30 h. Four of seven macaques with negligible disease already had detectable serum IFN by 6–12 h, and the remainder were positive by 24 h. Interferon was first detected at 24 h in all seven transiently ill monkeys. In the three monkeys that died from RVF virus, serum IFN was positive in only 1 at 24 h, and was not detected until 30 h in the other two. Furthermore, monkeys 4880 and 5606, initially positive in the 30 h sample, had only a transient, low-level IFN response in the face of substantial serum viremia. This strongly implicates a late and truncated IFN response as a mechanism leading to VHF. The disappearance of serum interferon concurrent with a persistent viremia suggests a failure to establish an effective antiviral state and may be a critical prognosticator of a fatal outcome. The third monkey (9C87) with lethal VHF had a relatively early (24 h) and vigorous IFN response that lasted throughout the course of viremia until death on day 6. This animal had a peak viremia of 7.4–7.5 $\log_{10}$ PFU/ml on day 1 and these levels were the highest measured in the study. Apparently, endogenous interferon failed to limit virus replication in target tissues as evidenced by the high, prolonged viremia concurrent with interferonemia.

The three monkeys that died presented similar clinical and anatomical pathological abnormalities. They had extensive, but not massive, liver necrosis, with the midzonal pattern characteristic of RVF virus in many species, including man [1, 2, 5, 6, 15, 30]. Although no intravascular fibrin thrombi were found, circulating fibrin split products, hypofibrinogenemia, thrombocytopenia, and other abnormalities of clotting [31] suggested the occurrence of disseminated

intravascular coagulation (DIC). Since most of these abnormalities, as well as a microangiopathic hemolytic anemia, were present by day 2 [31], a time which precedes extensive liver damage (Table 4), DIC may have been an important mechanism in disease induction. This suggestion is further supported by continued deterioration of monkey 5606, who died on day 15, even though viremia terminated on day 6. Liver disease may have contributed to hemorrhage and the expression of DIC through decreased synthesis of coagulation factors and through delayed clearance of activated coagulation factors and anticoagulant fibrin degradation products [32]. Liver necrosis may also have led to generation of local procoagulant activity with resultant activation of coagulation in hepatic vasculature [33]. The finding of intrahepatic sinusoidal thrombosis in cattle dying of RVF [34] emphasizes the need for further study of such mechanisms of disease production and aggravation in human and macaque infections.

The extent of hepatic involvement appeared to be a major clinical determinant of disease. Serum AST, ALT, and GGT values on day 2 or 3 were markedly elevated in fatally infected animals, whereas abnormalities in survivors were of much lesser magnitude. Sick animals destined to survive had somewhat higher values than those of monkeys without any overt clinical disease, although the differences were not great. Although the elevations of AST were disproportionate with respect to ALT and GGT we have observed a high degree of correlation between the extent of liver injury reflected in GGT and ALT values, the level of viremia, and the extent of hemostatic impairment reflected in PT and APTT values [31].

Cardiac involvement in monkeys with fatal VHF was signaled by the presence of focal myocarditis and the appearance of high levels of serum CK-MB. These findings may be secondary and are commonly seen in many VHF. The presence of a neutrophilic leukocytosis was a poor prognostic sign, another correlation seen in other VHF.

Thus, the IFN response during the first 24 h after RVF virus infection appears to be a major determinant of the outcome in rhesus macaques, just as early events seem to determine the outcome in rodents [12, 15]. The macaques, however, do not succumb with massive liver necrosis and untrammeled viral replication, as seen in genetically susceptible rats. The pathogenesis significantly affects the liver, but is more complex and may involve vascular damage as well. DIC appears to be an additional pathogenetic mechanism [10, 31].

Acknowledgement

The views of the author(s) do not purport to reflect the position of the Department of the Army or the Department of Defense.

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Received July, 21, 1989

Printed in Austria